

BRIEF COMMUNICATION

Molecular Cytogenetic Analysis of the Bladder Carcinoma Cell Line BK-10 by Spectral Karyotyping

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The bladder cancer cell line BK-10 was established from a grade III–IV transitional cell carcinoma (TCC). BK-10 is near-tetraploid ($\pm 4n$) and consists of two subclones with 20–25 structural aberrations. Here we report the cytogenetic analysis of BK-10 by G-banding, spectral karyotyping (SKY), and FISH. SKY refers to the hybridization of 24 differentially labeled chromosome painting probes and the simultaneous visualization of all human chromosomes using spectral imaging. SKY enabled us to confirm 12 markers in BK-10 previously described by G-banding, redefine 11 aberrations, and detect 4 hidden chromosomal rearrangements, 2 of which had been identified as normal or deleted copies of chromosome 20 and 1 as a normal chromosome 3. Twenty out of 21 translocations identified were unbalanced. FISH analysis of BK-10 using chromosome arm-specific paints, centromere probes, and oncogene/tumor suppressor gene-specific probes revealed a deletion of *CDKN2A* (*p16*) in all copies of chromosome 9, a low-level amplification of *MYC* (five copies), and loss of one copy of *TP53*; detected the presence of the Y chromosome in a hidden translocation; and detected four copies of *ERBB-2*. A probe set for *BCR* and *ABL* verified breakpoints for all translocations involving chromosomes 9 and 22. A new karyotype presentation, "SKY-gram," is introduced by combining data from G-banding, SKY, and FISH analysis. This study demonstrates the approach of combining molecular cytogenetic techniques to characterize fully the multiple complex chromosomal rearrangements found in the bladder cancer cell line BK-10, and to refine the chromosomal breakpoints for all translocations. *Genes Chromosomes Cancer* 25:53–59, 1999

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Bladder cancer is the sixth most common form of cancer in the United States, with an annual incidence of approximately 50,000 new cases. Transitional cell carcinomas (TCCs) comprise 90% of all reported cases; the remaining 10% are predominantly squamous cell carcinomas. Cytogenetic analysis of bladder carcinomas has thus far relied on conventional banding methods such as G-, Q-, and R-banding (Mitelman, 1994; Sandberg and Berger, 1994). Noninvasive grade I and II TCCs are primarily diploid ($2n$) with few chromosomal aberrations, whereas grade III and IV tumors exhibit numerous markers and an increase in ploidy. Non-random structural and numerical aberrations were observed involving chromosomes 1, 6, 7, 8, 9, 11, 13, 18, and chromosomal arms 3p, 5p, 10q, and 17p. Furthermore, 50% of all male bladder cancer patients were missing the Y chromosome. Molecular cytogenetic analysis utilizing fluorescence in situ hybridization (FISH), interphase cytogenetics, comparative genomic hybridization (CGH), and loss of heterozygosity analysis (LOH) has been applied to numerous TCC bladder tumors. DNA gains were mapped by CGH to chromosomes 7 and 10, as well as chromosome arms 1q, 5p, and 8q. DNA losses

have been reported for chromosome arms 1p, 3p, 4p, 6q, 8p, 9p, 9q, 11p, 13q, 17p, 18q, and the Y chromosome (for review see Fletcher et al., 1997).

The cell line BK-10 was established in 1995 from a grade III–IV, primary muscle-invasive transitional cell carcinoma of the bladder from a 67-year-old male. The cell line underwent serial passage for 20 months (36 passages) without exhibiting senescence. Our initial G-banding analysis revealed two major subclones that contained 20–25 structural chromosomal aberrations. Fourteen markers were common to both clones but each contained unique rearrangements, and 10 markers were not fully identifiable (Roberson et al., 1998).

In order to analyze the numerous chromosomal abnormalities of BK-10 more completely, we applied spectral karyotyping (SKY) analysis to G-banded metaphase chromosome preparations of

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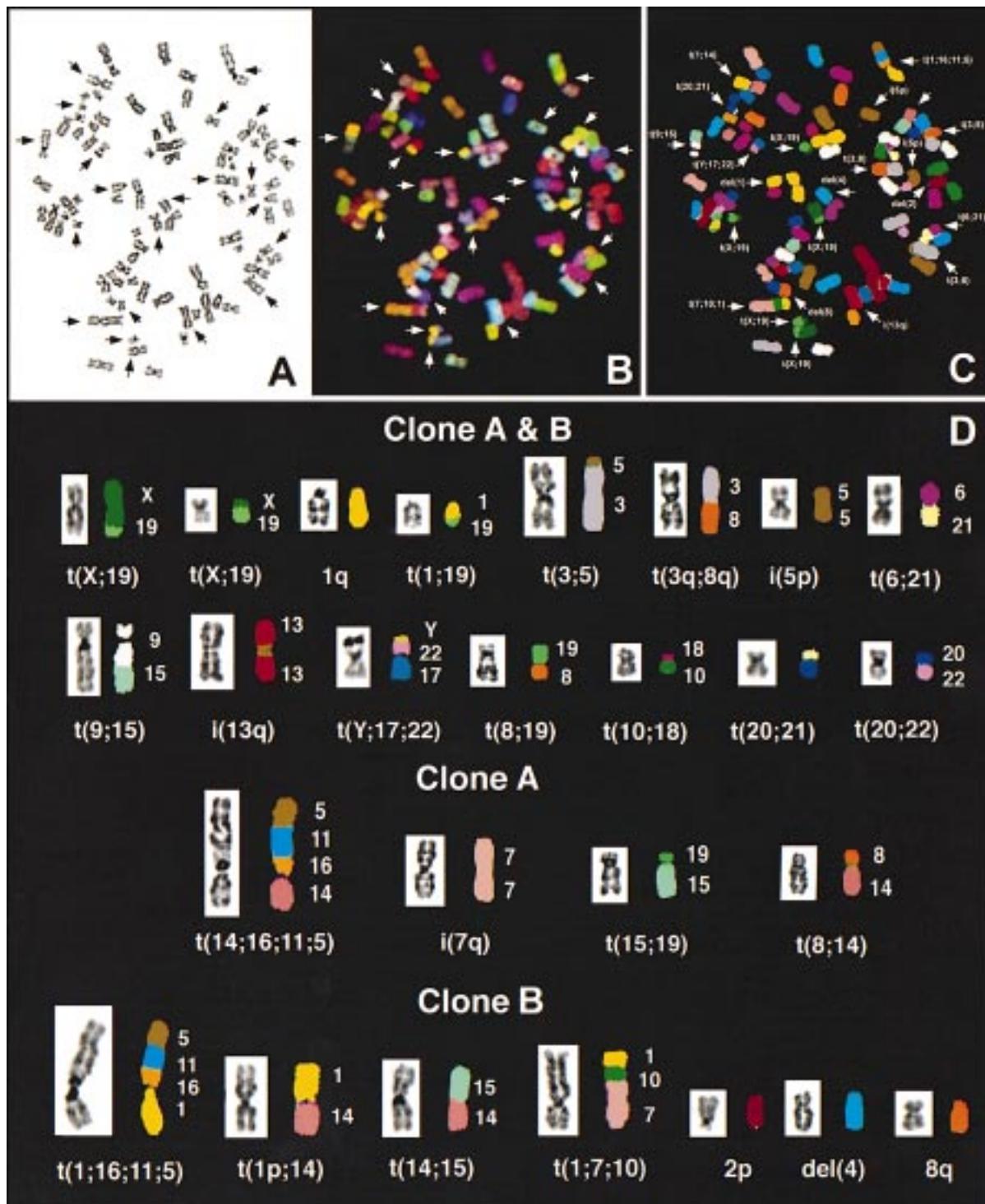


Figure 1. Spectral karyotyping analysis of BK-10. A representative BK-10 metaphase spread from clone B hybridized with SKY probes is depicted in panels A–C. The inverse-DAPI stained chromosomes are shown in A. The RGB image resulting from spectral karyotyping analysis (SKY) of the identical metaphase is shown in B; the arrows identify the

aberrant chromosomes. C shows the same metaphase spread as shown in A and B following chromosome classification. D presents a summary of the clonal aberrations found in both clones A and B and those unique to each subclone in the SKY classification pseudocolors (right), aligned next to their inverse-DAPI banded counterparts, depicted in gray scale.

this cell line. Hybridization with SKY probes resulted in the simultaneous color discrimination of all 24 human chromosomes. Visualization of the painted chromosomes is based on spectral imaging, a method combining fluorescence microscopy, Fourier spectroscopy, and CCD imaging (Garini et al., 1996; Schröck et al., 1996). SKY has become a valuable tool in cytogenetic analysis for both human and murine tumors (Liyanage et al., 1996; Schröck et al., 1996; Veldman et al., 1996; Macville et al., 1999).

BK-10 cells (passages 25 and 30) were harvested by mitotic "shake-off" and the cells were processed by standard cytogenetic methods (Modi et al., 1987). Hybridizations of BK-10 cells were performed on previously G-banded slides (at the 550-band-level stage) that had been stored at 40°C for 4 months. The G-banded metaphase slides were destained in 3:1 methanol:acetic acid, washed in 1 × PBS, and postfixed in 1% formaldehyde in 1 × PBS/50 mM MgCl₂ (10 min). G-banded metaphase chromosome preparations required 10–30 sec for denaturation in 70% formamide/2 × SSC at 80°C.

SKY painting probes were prepared from flow-sorted chromosomes (kindly supplied by Dr. Johannes Wienberg and Professor Malcolm Ferguson-Smith). Details of the labeling, hybridization, and detection protocols used were described in Macville et al. (1997). Image acquisition for SKY was performed as previously described (Schröck et al., 1996). Twenty SKY images with their companion inverted-DAPI images were analyzed and compared to 20 G-banded karyotypes prepared in our initial study (Roberson et al., 1998).

Metaphase cells from BK-10 were hybridized with painting probes for chromosomes 8, 9, 14, 17, X and Y, chromosome arm paints for 1q, 5q, 8q, 11p, 11q, 14q, 16q, 19p, X, and Yqh (arm paints kindly supplied by Dr. Michael Bittner) and centromere specific probes for chromosomes 1, 8, 14, and 17 (Vysis, Downers Grove, IL) for confirmation of the SKY results. Probes for the tumor suppressor gene *CDKN2A* (*p16*) (9p21; kindly supplied by Dr. Shiv Srivastava), the oncogenes *MYC* (8q24), *ERBB-2* (17q11.2), and *BCR* and *ABL* (22q11 and 9q34, respectively) (Vysis) were used to establish the presence or loss of these genes and to verify breakpoints within translocations established by the combined G-banding and SKY analysis.

BK-10 consists of two major subclones, both of which are near-tetraploid ($\pm 4n$), with chromosome numbers ranging from 83 to 102. G-banding analysis of BK-10 identified 15 translocations in clone A and 18 in clone B. Multiple copies of chromosomes 9 and 20 were observed (Roberson et al., 1998).

TABLE 1. Karyotypes of BK-10 Subclones^a

Clone A
83–102(4n), t(X;19)(q27;p13)x3,del(1)(p12), der(1) t(1;19) (p1?1;q13.?)del(1)(p21.?1), -2,der(3)
t(3;5)(p25?), i(5)(p10)x3,der(6;21)(p10;q10),i(7)(q10), +der(8)t(3;8)(p11;q11.2)x2,+9,+der(9)t(9;15)(q34;q12), der(10;18)(p10;p10)x2,i(13)(q10), der(14)
t(14;16;11;5)(p11;q11.2q24;q13q25;q13)x2,der(14)
t(8;14)(p21?:p12), der(15)t(15;19)(q10;p10),der(17)
t(Y;17;22)(q12;p11.2;q11.1)del(Y)(q11.2), der(19)
t(8;19)(q22;q13.1)x2,+20,der(20;21)(q10;q10)del(21)(q22.1), der(20;22)(p11.2;p11.2).
Clone B
83–102(4n), t(X;19)(q27;p13)x2,del(1)(p12), der(1)t(1;19) (p1?1;q13.?)del(1)(p21.?1), +der(1)t(1;16;11;5)
(p12;q11.2q24;q13q25;q13),der(1;14)(p13;p11), del(2)(q12),der(3)t(3;5)(p25?),del(4)(q23),i(5)(p10)x3, der(6;21)(p10;q10),der(7)t(1;7;10)?;p22;q21q26), del(8)(p12),+der(8)t(3;8)(p11;q11.2)x2,+9,+der(9)
t(9;15)(q34;q12), -10, der(10;18)(p10;p10)x2,i(13)(q10), -14,-15,der(14;15)(p11;q11.2),-16,der(17)t(Y;17;22)
(q12;p11.2;q11.1)del(Y)(q11.2), der(19)t(8;19) (q22;q13.1)x2,+20,der(20;21)(q10;q10)del(21)(q22.1), der(20;22)(p11.2;p11.2).

^aKaryotype nomenclature is according to ISCN (1995). The chromosomal rearrangements redefined by SKY are highlighted in bold print.

We analyzed images of previously G-banded metaphases from BK-10 by SKY and inverted-DAPI to clarify the numerous complex translocations and to uncover additional hidden aberrations. The complementary use of G-banding and chromosome painting not only revealed previously unrecognized chromosomal aberrations but greatly enhanced the ability to define chromosomal breakpoints in complex translocations. Figure 1A–C displays an inverted-DAPI image, the RGB display image, and the classified SKY image of a representative metaphase spread from clone B following hybridization with SKY painting probes. The inverted-DAPI banded image (Fig. 1A) has a banding pattern nearly identical to G-banding with two exceptions: first, there are fewer differences in intensity between dark and light bands, and second, unlike G-banded chromosomes, DAPI stains the heterochromatic regions more intensely. Figure 1D presents the clonal structural aberrations, common and unique to each clone. Table 1 summarizes the karyotypes of subclones A and B in BK-10. SKY confirmed the cell line as being near-tetraploid. Novel translocations or those redefined by the SKY analysis are highlighted in bold type.

SKY identified all numerical and structural aberrations. In clone A, 19 markers were detected and 9 were redefined. SKY analysis of clone B revealed 22 markers; 8 were redefined. Of note, four small metacentric chromosomes, found in both clones,

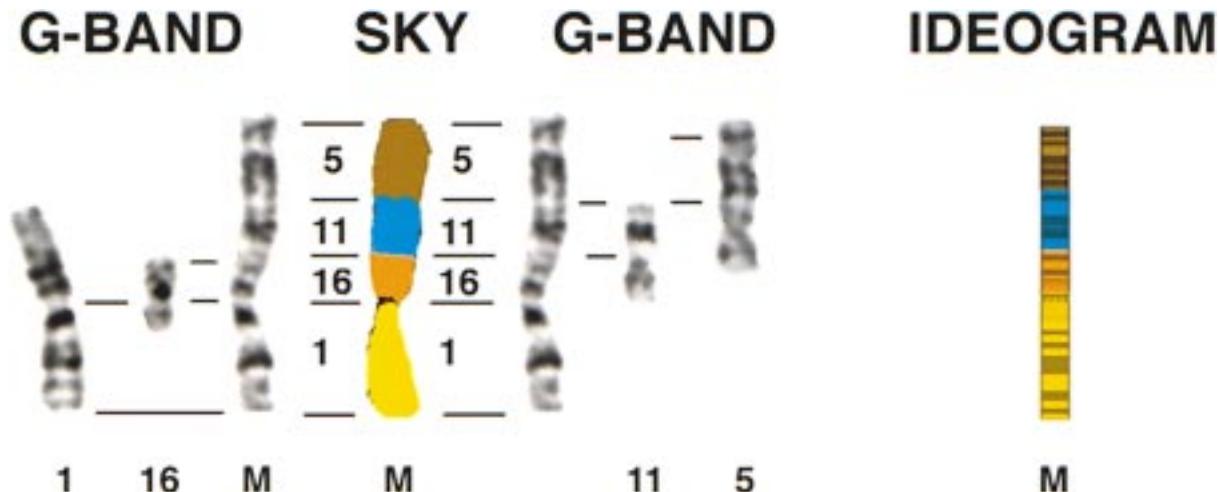


Figure 2. SKY ideogram reconstruction of marker $\text{der}(1)\text{t}(1;16;11;5)$. This figure exemplifies how combined G-banding and SKY analyses resolved the origin of the complex 1q marker chromosome present in clone B. By aligning the G-banded image and the SKY classified image next to each other, the individual segments of the marker are determined. These consisted of chromosomes 1 (yellow), 5 (brown), 11 (turquoise), and 16 (orange). Comparing the SKY marker with the

G-banded images of the normal chromosomes 1, 5, 11, and 16, the specific regions and breakpoints associated with this translocation were identified. Centromere-specific painting probes were used to determine which chromosome contributed the centromere in the marker. Finally, a colored ideogram of the marker was constructed, depicting the chromosome regions involved using the SKY classification colors (far right).

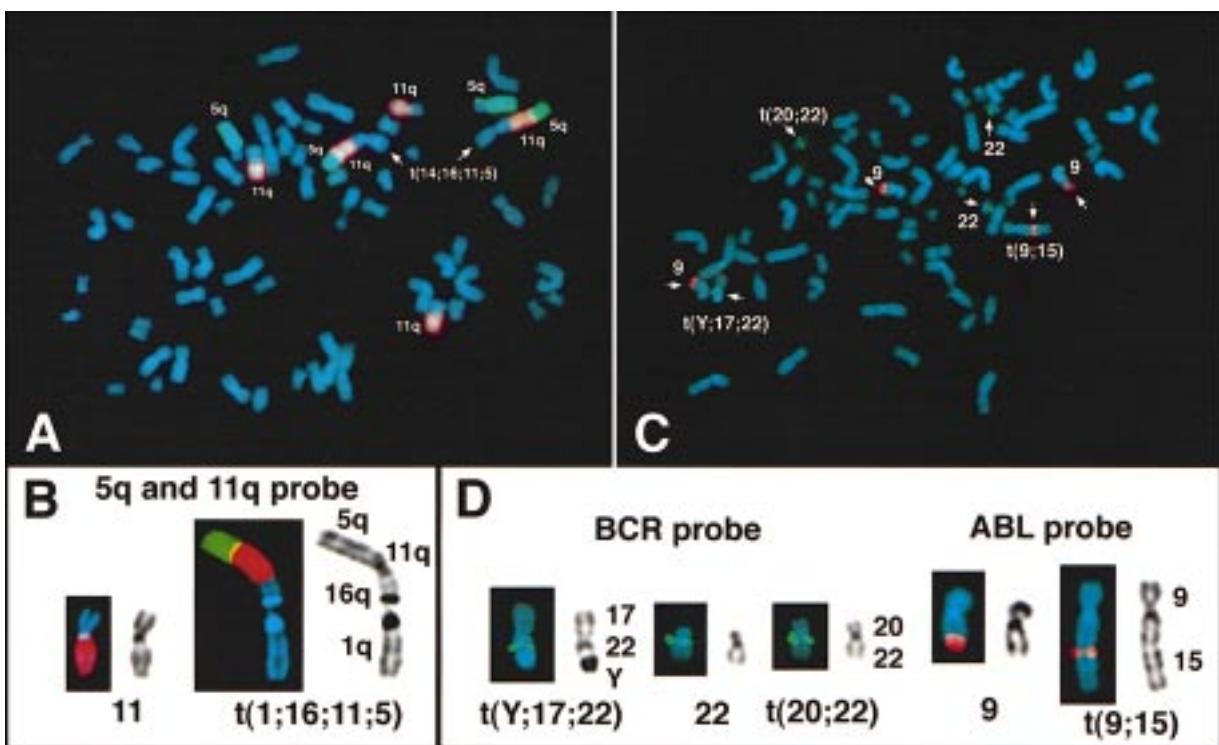


Figure 3. FISH probes for confirmation of SKY analysis of BK-10. A: BK-10 metaphase spreads from clone A were hybridized with chromosome arm paints for 5q (green fluorescence) and 11q (red fluorescence). These arm paints identified two normal chromosomes 5, three normal chromosomes 11, and two copies of the complex marker $\text{der}(14)\text{t}(14;16;11;5)(\text{p}11;\text{q}11.2\text{q}24;\text{q}13\text{q}25;\text{q}13)$ (compare also Fig. 2). B: Using the same approach as shown in A, a related marker was identified in clone B as

$\text{der}(1)\text{t}(1;16;11;5)(\text{p}11;\text{q}11.2\text{q}24;\text{q}13\text{q}25;\text{q}13)$ (compare also Fig. 2). C: Gene-specific DNA probes for the oncogenes *BCR* (located at 22q11.2, green signal) and *ABL* (located at 9q34, red signal) were hybridized to BK-10 cells to further delineate the breakpoints of chromosome translocations associated with chromosomes 9 and 22. These markers and examples of the normal chromosomes 9 and 22 are shown in D and are fully described in Table 1.

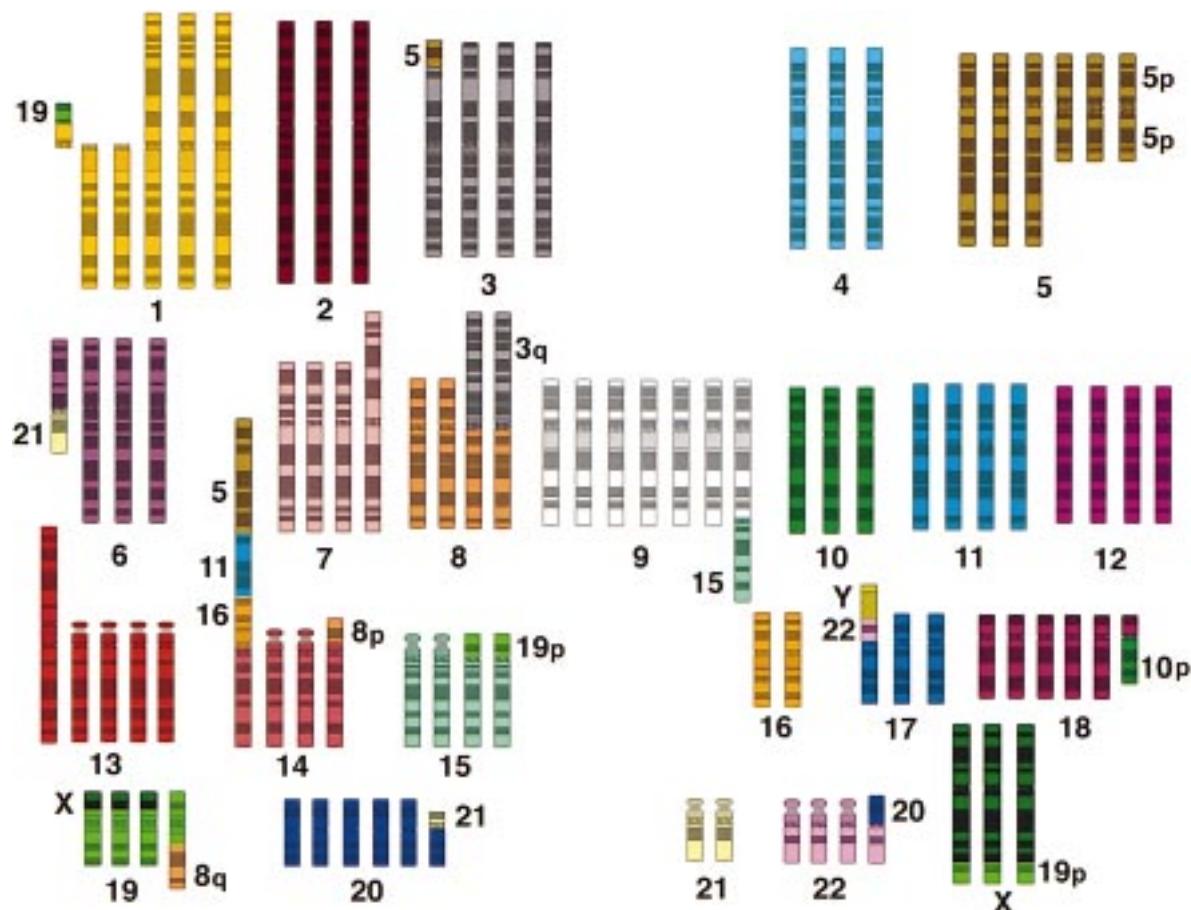


Figure 4. SKY-gram for BK-10. Karyotype of a representative metaphase spread from clone A in BK-10 consisting of colored ideograms. The colors used for this SKY-gram are the same as applied in the SKY classification visualizing the origin of the chromosomes involved in all the rearrangements.

which had been interpreted as "normal" chromosomes 20 and/or 3, were revealed as the translocations der(3)t(3;5), der(10;18), der(20;21), and der(19)(X;19). Consequently, the gain of chromosome 20 was less pronounced than we initially thought. The combined SKY, inverted-DAPI, and G-banding analysis of BK-10 revealed that 20 out of the 21 translocations were unbalanced translocations, resulting in copy number changes for many chromosomes and chromosome arms. Only t(X; 19)(q26;p12) was a balanced translocation. Fifty percent of the translocations were whole-arm translocations. Of note, neither monosomy 9 nor obvious 9p deletions, commonly observed in TCC, were present in BK-10. The cells contained multiple copies of chromosome 9 (5–8) and one copy of a derivative chromosome, der(9)t(9;15)(q34;q12). In order to determine the presence of small interstitial deletions in the short arm of chromosomes 9, we used dual-color FISH with a probe for the *CDKN2A* (p16) tumor suppressor gene and a chromosome 9

painting probe. Normal control metaphase cells clearly showed FISH signals for this gene; however, all copies of chromosome 9, including the translocation chromosome der(9)(9;15), failed to show a signal, suggesting that there were deletions of this tumor suppressor gene in all copies.

In our initial G-banding analysis we did not detect an intact Y chromosome. SKY analysis revealed a marker in both clones that involved 17q. DAPI staining showed a G-dark band at the distal end of the 17q marker, which suggested the presence of the heterochromatic region of the Y chromosome (Fig. 1A). SKY analysis disclosed that the marker contained predominantly heterochromatic material with a small amount of Y chromosome euchromatin. FISH performed with a Y chromosome painting probe and probe specific for the heterochromatic region confirmed that the heterochromatic portion of the Y chromosome was present in the translocation with the breakpoints at Yqter and Yq11.2.

The value of our SKY analysis has been greatly enhanced by assessing the information pertaining to chromosomal breakpoints derived from the G-banding analysis. The potential of integrating banding and painting information is presented in Figure 2 for clone B using the large der(1q) marker as an example (Table 1). In order to determine breakpoints of the aberrations, inverted-DAPI chromosomes were aligned next to their respective SKY classified images and subsequently compared to their G-banded counterparts, taken from images produced in the previous G-band analysis (Robertson et al., 1998). The origin of all components of this marker could not be accurately identified with G-banding alone. SKY and inverted-DAPI images revealed that the long arm was derived from chromosome arms 16q, 11q, and 5q, with 16q located at the proximal end and 5q at the distal end. Once the chromosomal origins of all portions of this marker chromosome were determined, a comparison of the banding patterns within the long arm was made to the normal G-banded chromosomes 1, 16, 11, and 5. The presence of 5q and 11q material in both der(14)t(14;16;11;5) and der(1)t(1;16;11;5) was confirmed by chromosome arm painting probes as shown in Figures 3A and B.

This alignment of SKY images with G-banded chromosomes and homologous chromosomes involved in translocations enhanced our ability to map chromosomal breakpoints with greater confidence. Confirmation of SKY analysis for very small chromosome segments lacking identifiable bands was made using chromosome arm specific paints and loci specific probes, for example, translocations t(X;19), der(8;19), der(17)t(Y;17;22), and der(20;22). Information regarding total copy numbers of the oncogenes *MYC*, *ERBB-2*, *BCR*, *ABL*, and the tumor suppressor gene *TP53* as confirmed by FISH analysis, is presented in Table 2 and demonstrated in Figures 3C and D.

The final results of the molecular cytogenetic analysis for clone A are displayed as a composite chromosome ideogram superimposed with the SKY classification colors in Figure 4. The information provided by this combined analyses enables one to construct a "SKY-gram" to visualize in a rapid and efficient manner the structural and numerical aberrations found in solid tumors containing multiple complex rearrangements such as those observed in BK-10.

The copy numbers of translocation chromosomes varied; for example, the der(9)t(9;15) occurred only

TABLE 2. Summary of FISH Mapping of Oncogenes and Tumor Suppressor Gene *CDKN2A* (*p16*)

Gene name	Presence (+/-)	Number of copies	Location
<i>MYC</i>	+	5–6	8q24 normal 8 (2), der (3q;8q) (2), der(19)t(8;19), del(8)
<i>BCR</i>	+	4–5	22q11 normal 22 (2–3), der(22)t(20;22) der(17)t(Y;17;22)
<i>ABL</i>	+	5–9	9q34 normal 9 (5–8) der(9)t(9;15)
<i>CDKN2A</i> (<i>p16</i>)	–	0	9p215
<i>TP53</i>	+	3	17p13 normal 17 (2) der(17)t(Y;17;22)
<i>ERBB-2</i>	+	4	17q11.2 normal 17 (3) der(17)t(Y;17;22)

once, whereas the translocations t(3;8) and der(1)t(1;16;11;5) and the isochromosome i(5p) were present in multiple copies. We conclude that these latter aberrations occurred early in tumor development because they precede the tetraploidization that occurred in BK-10. Therefore, we hypothesize that the acquisition of extra copies of chromosome arms 8q, 5p, 1q, as well as the loss of sequences on 3p and 8p were early events in bladder carcinogenesis. Interestingly, only 1 of the 21 translocations, the t(X;19)(q26;p12), was a balanced translocation. All other translocations were unbalanced, i.e., resulted in copy number changes of the chromosomes and chromosome arms involved. In addition to unbalanced structural translocations, aneuploidy of chromosomes and isochromosome formation contributed to copy number changes.

Surprisingly, there were no copy number losses for chromosome 9 in either subclone, and in particular, chromosome 9p deletions were not immediately obvious in this cell line, even though this particular chromosomal aberration is considered a landmark cytogenetic abnormality in bladder carcinomas. It has been reported that in earlier stages of bladder cancer, monosomy or deletions of chromosome 9 were present in approximately 70% of all primary bladder tumors (Fletcher, 1997). There is evidence that there are two important tumor suppressor loci involved with TCC on chromosome 9, one near band 9p21, the location of the *CDKN2A* (*p16*)

inhibitor tumor suppressor gene, and the other at 9q34. BK-10 contains multiple copies (>5) of chromosome 9, resulting in an overall gain for chromosome 9. It is remarkable that *CDKN2A* was deleted in all copies of chromosome 9 and in the translocation der(9)t(9;15)(q34;q12). However, as revealed by FISH mapping of the *ABL* oncogene (located at 9q34), all normal 9s as well as the der(9)t(9;15) contained this oncogene, suggesting that if there is a tumor suppressor gene located in 9q, it is located distal to this oncogene.

By combining banding and SKY analyses we redefined more than half of the translocations described by G-banding. In all instances, the break-points of chromosomal translocations were identified by comparing the banding and painting patterns and confirmed for many translocations using FISH analysis (Table 2). This approach of combining the SKY and G-banding chromosomal analysis overcomes two important hurdles, namely, the identification of hidden translocations and the reconstruction of complex rearrangements.

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